Advances in Brief

KAI1 Protein Is Down-Regulated during the Progression of Human Breast Cancer[1]

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Abstract

The *KAI1* gene was identified as a metastasis suppressor gene for human prostate cancer. Recently, we showed that *KAI1* mRNA levels were higher in an immortal, normal-like breast epithelial cell line and nonmetastatic breast cancer cell lines but lower substantially in highly metastatic breast cancer cell lines. In this study, we examined KAI1 protein expression in breast cancer cell lines by Western blot and immunohistochemical study. KAI1 protein levels paralleled *KAI1* mRNA levels and were inversely correlated with the metastatic potential of breast cancer cells. Furthermore, we examined KAI1 protein expression immunohistochemically in specimens from 81 patients with breast cancer and then correlated the findings with the clinical and histopathological parameters of the patients. High levels of KAI1 protein expression were found in normal breast tissues and noninvasive breast cancer (ductal carcinoma *in situ*). In contrast, KAI1 expression was reduced in most of the infiltrating breast tumors. We found that, in general, more malignant tumors demonstrated significantly lower KAI1 expression ($P = 0.004$). Additionally, among 29 specimens demonstrating multiple stages of malignancy within a single specimen, 23 demonstrated significant differences in KAI1 expression between benign breast tissue, ductal carcinoma *in situ*, and invasive carcinoma. The higher the incidence for malignancy within a given specimen, the lower the KAI1 expression ($P < 0.001$). These data suggest that in advanced breast cancer, KAI1 expression is down-regulated. Therefore, KAI1 may be a potentially useful indicator of human breast cancer progression.

Introduction

Breast cancer is the second most common cause of cancer-related death among women in the United States (1). As with other cancers, metastasis in breast cancer is the leading cause for mortality. Breast cancer is genetically heterogeneous, and a variety of genetic lesions have been identified that may contribute to disease progression (2). Chromosome 11, in particular 11p, is one of the most common regions undergoing genetic alterations in breast cancer (3–5). Introduction of a normal human chromosome 11 into a highly metastatic breast cancer cell line, MDA-MB-435, dramatically reduced the numbers of lung metastases in nude mice (6). This suggests that human chromosome 11 harbors a metastasis suppressor gene for human breast cancer.

KAI1, mapping to chromosome 11p11.2, was identified as a metastasis suppressor gene for human prostate cancer (7). Expression of KAI1 in a highly malignant prostate cancer cell line resulted in a significant suppression of lung metastases. KAI1 encodes a protein of 267 amino acids with a molecular weight of 29,610 (7). KAI1 is identical to CD82, which is a member of the TM4SF[4] (7). The TM4SF proteins share similar structures by containing four highly conserved hydrophobic regions, presumed to be transmembrane domains. The precise biochemical functions of TM4SF proteins are not known; however, current data suggest largely that they are involved in the regulation of cell development, proliferation, activation, and motility (8). At least three different TM4SF proteins, ME491/CD63, MRP-1/CD9, and KAI1/CD82, have been implicated to play important roles in tumor progression and metastasis (8). The role of KAI1 in cancer progression appears not to be limited to prostate cancer only. After its initial identification, KAI1 has been shown to be involved in the progression of human pancreatic cancer, non-small cell lung cancer, bladder cancer, breast cancer, and gastric cancer (9–13).

In our previous study, we compared the metastatic propensity and invasive ability of a continuum of breast cancer cells with varying degrees of progression toward malignancy and found that these parameters appeared to correlate inversely with *KAI1* mRNA expression (14). The purpose of this study was to determine whether there exists a correlation between the loss of KAI1 protein expression with advancing breast cancer disease. To test this, we examined KAI1 protein levels by Western blot in four breast cancer cell lines representing different stages of progression. In addition, we performed immunohistochemical studies to assess KAI1 protein expression in 81 human breast cancer specimens from patients with known clinical outcome. Our results indicate that KAI1 protein expression decreased in

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[4] The abbreviations used are: TM4SF, transmembrane 4 protein superfamily; DCIS, ductal carcinoma *in situ*.

metastatic breast cancer cell lines and in patients with highly malignant breast cancer.

Materials and Methods

Cell Lines and Culture Conditions. The immortal, normal-like human breast MCF-10A cell line (provided by Dr. Robert Soule, Karmanos Cancer Center, Detroit, MI) was grown in DMEM/Ham's F-12 media supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 12 μg/ml insulin, 0.02 ng/ml epidermal growth factor, and 1 ng/ml cholera toxin (15). Human breast cancer cell lines MDA-MB-231 and T47D were obtained from The Lombardi Cancer Center Tissue Culture Core Facility. MDA-MB-435 cells were a gift from Dr Janet Price (University of Texas M. D. Anderson Cancer Center; Ref. 16). All breast cancer cell lines were maintained in modified improved MEM containing 10% fetal bovine serum. All cells were incubated at 37°C in 5% CO_2 in a humidified chamber. All cell lines were routinely tested and found to be negative for *Mycoplasma* contamination.

Western Blot Analysis. Cell proteins were solubilized in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl, 1 mM EDTA, 1% Triton X-100, and 10% glycerol] containing protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and trypsin inhibitor). Twenty μg of cell lysate were mixed with Laemmli's sample buffer without 2-mercaptoethanol and boiled for 5 min. After SDS-PAGE (17.5%; Norvex, San Diego, CA), proteins were electrophoretically transferred to nitrocellulose (Amersham, Arlington Heights, IL) and probed with C33, a specific monoclonal antibody against KAI1 (a gift from Dr. Osamu Yoshie, Shionogi Institute for Medical Science, Osaka, Japan; Ref. 17). An enhanced chemiluminescence (ECL; Amersham) system was used for signal detection.

Pathological Specimens. Eighty-one surgically resected primary cancer tissues were obtained from patients treated at The Lombardi Cancer Center. All tissues used in this study are from the Lombardi tumor bank, and they include: (*a*) normal breast tissue from patients with breast cancer; (*b*) samples from patients with DCIS; and (*c*) samples from patients with invasive breast carcinoma.

Immunohistochemistry. Immunohistochemical staining was done on frozen tissue sections by a typical immunoperoxidase method. Briefly, 6-μm frozen sections were cut on a cryostat and fixed with acetone for 10 min at 4°C and air-dried. All subsequent steps were performed at room temperature. The sections were incubated with 10% fetal bovine serum for 1 h and then treated with 10% H_2O_2 for 5 min to block the endogenous peroxidase activity. The sections were then incubated with the mouse monoclonal antihuman KAI1 antibody (anti-CD82; PharMingen, San Diego, CA) at a dilution of 1:150 for 2 h in a moist chamber. After treatment with biotinylated rabbit anti-mouse immunoglobulin (1:250) for 1 h, the sections were incubated with aminoethyl carbazole containing 0.1% H_2O_2 for 1 h at 37°C. The sections were counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene, and mounted with Permount.

Staining intensity in the cancer cells was estimated as positive when it appeared to be similar to that of normal breast duct cells and benign fibroadenoma tumor cells. When the percentage of KAI1-positive cells within a particular type of tissue was 51% or greater, the specimen was classified as KAI1 abundant. When 5–50% or 0–4% of cells were positively stained with KAI1, the sample was classified as decreased or negative, respectively. Twenty-nine samples contained multiple types of tissue within one specimen. Each tissue type was scored for KAI1 expression separately, based on its staining intensity and pattern.

Statistical Analysis. To describe the relationship between pathological severity and immunohistochemical detection of KAI1, the worst pathological characteristic and its corresponding KAI1 expression level were used to classify each individual. This relationship was assessed by The Jonckheere-Terpstra test (18). For specimens with multiple types of tissue present, the sign test was performed using the worst and the best tumor characteristics to determine whether KAI1 expression was related to tumor severity within the same person (19). Because two statistical tests were performed with the same set of specimens, a Bonferroni correction was used so that each test was significant if $P < 0.025$. Survival status information is available for 72 cases, including 27 deaths. Survival curves and the median follow-up time among survivors were estimated according to the methods of Kaplan and Meier (20). Log-rank tests were performed to determine whether patients with negative KAI1 expression demonstrated a different survival profile than patients with any KAI1 expression.

Results

Previously, we found that *KAI1* mRNA levels were inversely correlated with the metastatic potential of some breast cancer cells (14). In this study, we performed Western blot analysis and immunohistochemistry to examine KAI1 protein levels in some of these breast cancer cells as well as an immortalized breast epithelial cell line, MCF-10A. MCF-10A cells are not tumorigenic (15). T47D, a breast cancer cell line, is neither invasive *in vitro* nor metastatic *in vivo*. MDA-MB-231 and MDA-MB-435 are highly malignant breast cancer cell lines (21).

Western blot analysis was performed using a specific monoclonal antibody (C33) against KAI1 (17). The molecular weight of KAI1 protein in these cells ranged from 46,000 to 60,000, probably because of its glycosylation (Fig. 1). MDA-MB-435, which is highly metastatic, had an extremely low level of KAI1 protein. MDA-MB-231 cells, reported to be highly invasive but modestly metastatic in athymic nude mice (16), had higher levels of KAI1 protein than MDA-MB-435 cells (Fig. 1). A nonmetastatic breast cancer cell line, T47D, had much higher KAI1 protein than metastatic MDA-MB-435 cells. In addition, MCF-10A, a nontumorigenic breast epithelial cell line, had the highest protein levels among all of these cells. Taken together, these studies indicated that KAI1 protein expression also was inversely correlated with the metastatic potential of these breast cancer cells.

To determine whether there is a correlation between the loss of KAI1 expression and breast cancer progression and to test the feasibility of using KAI1 expression as a marker of breast cancer metastases, we examined KAI1 expression immu-

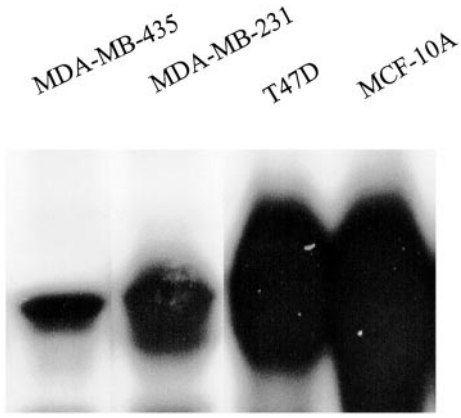

Fig. 1 Western blot analysis of KAI1 protein expression in some established breast cancer cells. Proteins were extracted from subconfluent monolayer cell culture and separated by SDS-PAGE and then transferred to nitrocellulose membrane. The blot was probed with C33, a specific monoclonal antibody against KAI1 (a gift from Dr. Osamu Yoshie, Osaka, Japan). Enhanced chemiluminescence (ECL) was used for signal detection.

nohistochemically in specimens from 81 patients with breast cancer. All specimens were graded by pathologists and classified into three groups: group 1, normal and benign lesions; group 2, DCIS; and group 3, infiltrating cancer (including moderately differentiated and poorly differentiated invasive cancers). Twenty-nine specimens had two or more types of cells within one specimen. For analysis, each specimen was graded by its most malignant cell type and scored by KAI1 expression. Significantly high levels of KAI1 expression were found in normal breast tissues and benign breast tumors from patients with breast cancer. There also was abundant KAI1 staining in the patients at the earlier malignant tumor stage (DCIS). However, KAI1 expression was dramatically reduced in most of the infiltrating breast tumors (Table 1). Furthermore, poorly differentiated tumors had much less KAI1 protein than well and moderately differentiated ones (Fig. 2). In general, more malignant tumors demonstrated significantly lower KAI1 expression ($P = 0.004$). Of the 29 specimens containing a spectrum of tissue types, 23 demonstrated differences in KAI1 expression. The more malignant cells within individual specimens showed significantly lower KAI1 expression than less or nonmalignant cells in the same individual ($P < 0.001$). To avoid selection bias, each slide was also graded by its least malignant cell type and scored for KAI1 expression. The statistical analysis indicated the same trends of lower KAI1 expression among increasingly malignant cells. The effect of KAI1 on overall survival of these patients was analyzed to determine whether KAI1 expression contributes to the prognosis of breast cancer. Survival status information was available for 72 patients, including 27 deaths. The survival curves for individuals with any KAI1 expression compared with negative KAI1 expression are shown in Fig. 3. KAI1 expression appears to be associated with better survival. The survival estimates at 3 years are 80% (SE, 7%) and 74% (SE, 8%) for those individuals with any KAI1 expression *versus* negative expression, respectively. By 5 years, the survival estimates are 70% (SE, 8%) and 57% (SE, 9%) for any and negative KAI1 expression groups, respectively. However, the difference in the curves did not reach statistical significance ($P = 0.180$). The median follow-up time among survivors is 5.9 years.

Table 1 Frequencies of KAI1 expression in breast cancer patients

More malignant tumors demonstrated a significantly lower expression of KAI1 ($P = 0.004$). Of 29 specimens demonstrating multiple stages of malignancy, 23 displayed differences in KAI1 expression. The higher the incidence for malignancy within an individual, the lower the KAI1 expression. Specimens with low or no incidence for malignancy had higher KAI1 expression ($P < 0.001$).

		KAI1 Expression		
Tissue type	Cases examined *n*	Abundant *n* (%)	Decreased *n* (%)	Negative *n* (%)
Normal	7	4 (57%)	1 (14%)	2 (29%)
DCIS	7	5 (71%)	1 (14%)	1 (14%)
Cancer	67	13 (19%)	19 (28%)	35 (52%)

Discussion

Metastasis is a complicated multistage process that requires the coordination of multiple genes, including both metastasis stimulating genes and metastasis suppressor genes (22). Genomic instability is one of the driving forces for tumor progression and metastasis development. Among all genetic alterations, inactivation of metastasis suppressor genes is one important factor contributing to the formation of tumor metastasis. Chromosome 11, in particular 11p, is one of the most common regions undergoing genetic alterations in human breast cancer (3–5). A previous study demonstrated that a breast cancer metastasis gene or genes exists on chromosome 11 by the fact that the introduction of a normal copy of chromosome 11 into malignant breast cancer cells significantly suppressed their metastatic ability (6).

The *KAI1* gene, located on human chromosome 11p11.2, was initially identified as a metastasis suppressor gene for human prostate cancer (7). Down-regulation of the KAI1 protein was observed during the progression of prostate cancer (23). However, the role of KAI1 in tumor progression may not be limited to prostate cancer. KAI1 expression was reported to correlate with favorable outcome in patients with non-small cell lung cancer (10, 24). KAI1 expression also was reported to be reduced in metastatic human pancreatic cancers and high-grade bladder cancers by *in situ* hybridization studies (9, 11). Recently, White *et al.* (25) analyzed KAI1 protein in normal and cancer cells of a variety of tissues, and they found that KAI1 protein was down-regulated in most of the cancer cell lines analyzed. The role of KAI1 expression in gastric cancer seems to be controversial. One study showed that KAI1 was unchanged in metastatic and nonmetastatic gastric cancers (26), whereas a more recent study demonstrated that KAI1 expression was decreased in high-grade gastric cancer (13). In our previous study, we observed that *KAI1* mRNA levels were inversely correlated with the metastatic potential of some breast cancer cell lines (14). The present data showed that the expression of KAI1 protein was also down-regulated in highly malignant breast cancer cell lines and specimens. Although most nonma-

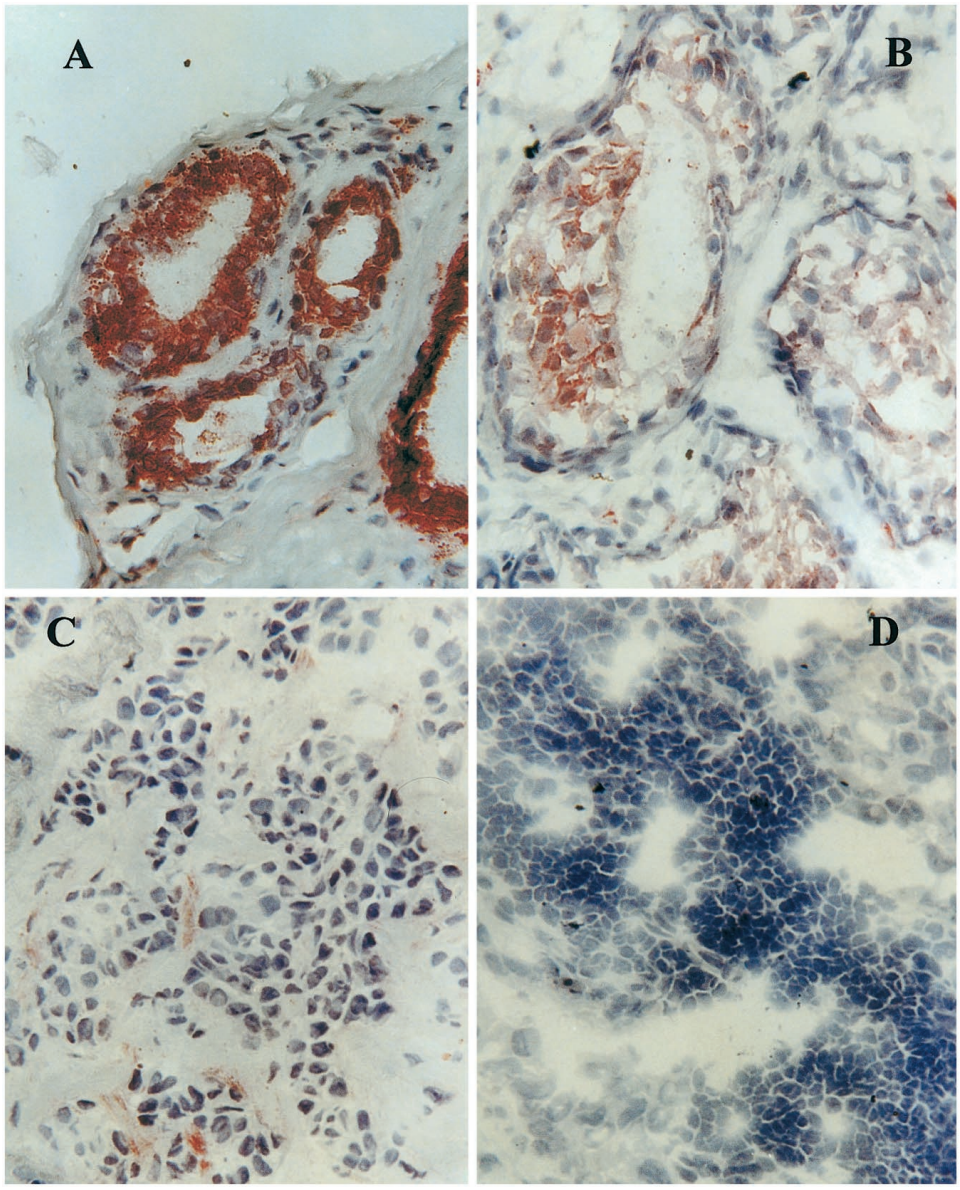

Fig. 2 Immunohistochemical staining of KAI1 protein in tissues of breast cancer patients using a mouse monoclonal antibody against KAI1 (anti-CD82; PharMingen) and aminoethyl carbazole as the chromogen with hematoxylin counterstaining. *A*, normal breast ducts showing abundant KAI1 protein at the cell-to-cell border. *B*, DCIS expressing moderate KAI1 protein. *C*, infiltrating breast cancer, which is well to moderately differentiated, having very weak KAI1 staining. *D*, negative staining of the tumor cells of the poorly differentiated breast carcinoma ×200.

lignant and DCIS tissues (9 of 14) tested were shown to be positive for the anti-KAI1 antibody, only 19% (13 of 67) of infiltrating tumor specimens retained strong KAI1 staining. Most strikingly, more pleomorphic tumor cells had significantly lower levels of KAI1 protein as compared with lower grade tumor or normal ductal cells in the same specimen. Therefore, within an individual, the KAI1 expression was also inversely correlated with the severity of tumor. In summary, our results, which are consistent with most of the current literature, support the role of KAI1 as a favorable prognostic factor for a variety of human cancers.

More recently, Huang *et al.* (12) investigated the correlation of reduction in KAI1 expression with recurrences in breast cancer patients. The results demonstrated that the disease-free survival rate of patients with KAI1-negative tumors was significantly lower than that of patients with KAI1-positive tumors. In their other study, they showed that KAI1 expression in non-small lung cancer is a favorable prognostic factor for both overall and disease-free survival (24). In our study, we also examined the relationship between KAI1 expression and overall survival status of these patients with breast cancer. Our data indicated that KAI1-positive patients tended to have better overall survival rate as compared with KAI1-negative patients. However, the difference is not statistically significant (Fig. 3). This result is consistent with Huang *et al.*, who demonstrated that KAI1 expression is associated with disease-free survival ($P = 0.0065$) but not with 5-year survival ($P = 0.3080$; Ref. 12). Whether KAI1 is a better predictor for breast cancer recurrences than overall survival remains to be seen in a much larger study popula-

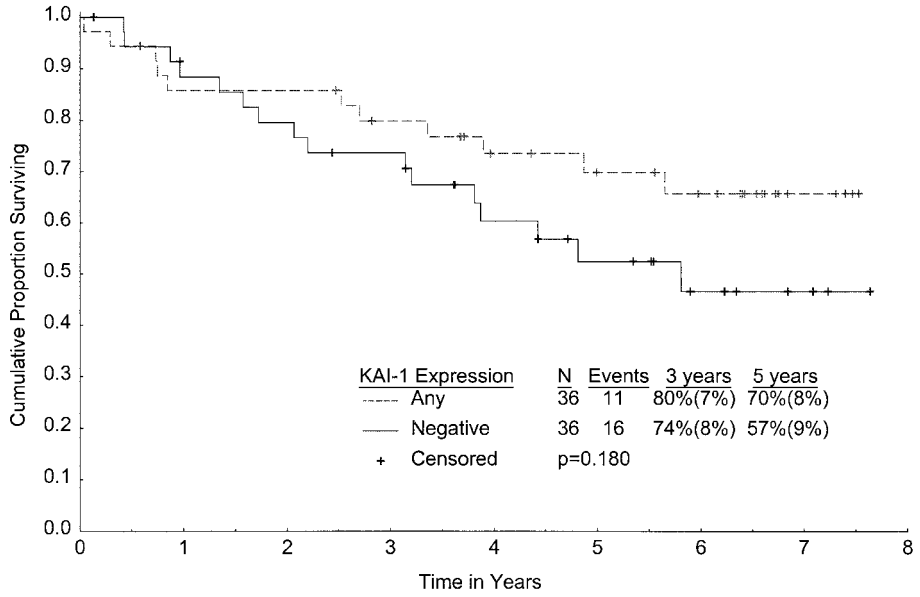

Fig. 3 Survival of patients with negative KAI1 expression (0–4%) *versus* abundant or decreased KAI1 expression (>4%). Survival estimates at 3 years and at 5 years are provided with SEs in parentheses.

tion. In addition, Huang's study examined the combined effect of KAI1 and CD9 on breast cancer recurrences and survival. Their results indicated that the disease-free survival and 5-year survival rate of patients with either CD9-negative or KAI1-negative tumors were both significantly lower than those for patients who expressed these proteins. Multiple factors clearly contribute to breast cancer survival. Further study of the alterations in these genes may contribute more precise prognostication in breast cancer.

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